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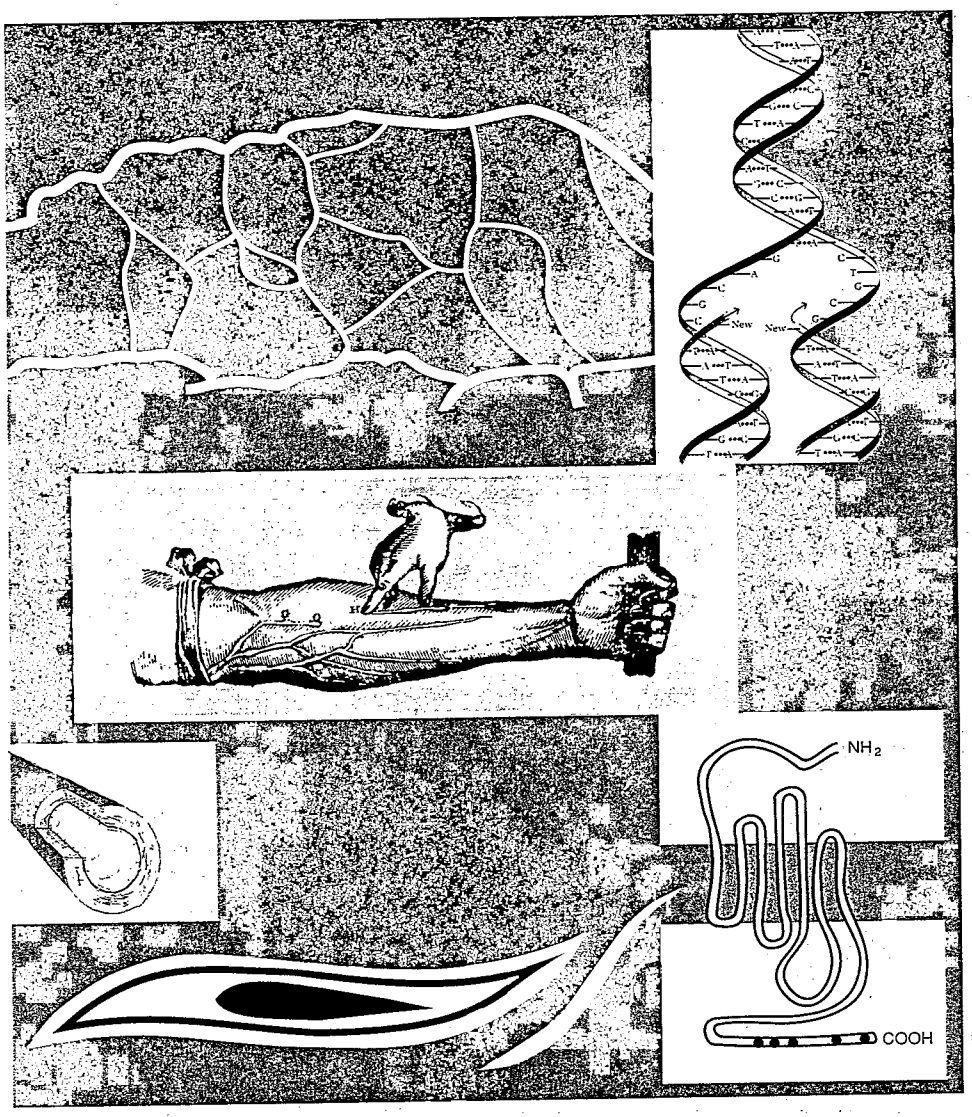
Journal of Vascular Research



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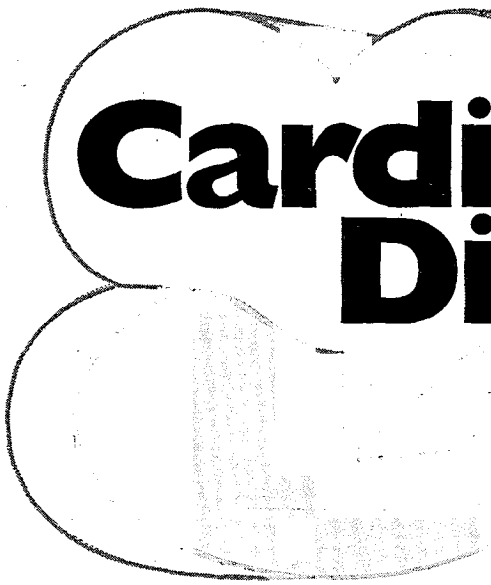

Editors: Mario Timio, Volker Wizemann

Main Headings

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Renal Disease and Systemic Disorders
The Heart in Replacement Therapy
Cardiorenal Dysfunction in Hypertension
Hypertension in Replacement Treatment

This volume is a valuable update on advances in the relatively new discipline of cardionephrology. Renowned specialists in the fields of cardiology, nephrology and hypertension provide an overview of the illnesses affecting both the heart and the kidney, and identify problems of common concern. Insights gained from the latest research into cardiorenal interaction are presented, and steps towards improving the diagnosis and management of renal disorders with cardiac involvement, and cardiac disorders involving the kidney, are discussed. By helping to elucidate the chain of events by which renal dysfunction disrupts the function of the heart, and vice versa, this book makes a worthwhile contribution to ongoing efforts to develop better diagnostic and therapeutic approaches.

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Research Paper

J Vasc Res 1994;31:247–255

Susan M. London
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Department of Neurological Surgery, University of Washington Medical Center, Seattle V.A. Medical Center, Seattle, Wash., USA

Kinetics of Bromodeoxyuridine Uptake by Smooth Muscle Cells after Arterial Injury

Key Words
Vascular smooth muscle
Bromodeoxyuridine
Cell cycle kinetics
Arterial injury

Abstract
The time course of 5-bromo-2′-deoxyuridine (BrdU) uptake by medial and neointimal smooth muscle cells was examined in rat carotid arteries at periods of 1–20 days after balloon catheter injury. DNA-incorporated BrdU was determined by the indirect immunoperoxidase technique in both plastic- and paraffin-embedded specimens. The BrdU labeling index (LI) peaked at 48 h in the media (19.9% paraffin, 9.4% plastic) and at 5 days in the neointima (55.4% paraffin, 60.2% plastic). Immunohistochemistry for the endogenous marker proliferating cell nuclear antigen was variable and generally less reliable than BrdU. Using total viable medial smooth muscle cell number as an index of medial injury, the LI for BrdU was directly proportional to the extent of injury at 48 h, and correlated with loss of actin immunoreactivity. BrdU immunohistochemistry is a reliable alternative to [^{3}H]-thymidine autoradiography for short-term labeling in this model. Proliferation of smooth muscle cells in this model is likely related to the degree of injury to the media.

Introduction

Smooth muscle cell (SMC) proliferation has been implicated in atherogenesis [1] and is a common sequel to arterial trauma such as graft placement, carotid endarterectomy and balloon catheter angioplasty [2]. Mechanisms related to hyperplasia of vascular SMC remain poorly understood; studies of SMC growth kinetics have proven valuable in the characterization of this phenomenon.

Incorporation of radiolabeled thymidine ([^{3}H]-thymidine) is a widely employed technique for labeling cells in the synthetic (S) phase of the cell cycle, and has been used in vivo [3–11] and in vitro [12]. Halogenated pyrimidines such as bromodeoxyuridine (BrdU) are incorporated into dividing cells at the time of DNA doubling via the salvage pathway [13]. Monoclonal antibodies which discriminate between halogenated pyrimidines and thymidine [14, 15] offer a rapid, cost-effective alternative for the study of cytokinetics [16]. Like [^{3}H]-thymidine, BrdU can be used in vivo [16–19] and in vitro [20]. The sensitivity [14, 21–23] and specificity [22] of BrdU immunodetection for labeling S-phase cells are comparable to [^{3}H]-thymidine autoradiography.

Immunohistochemical detection of endogenous cell cycle-specific proteins has also been employed to evaluate cell proliferation. These markers include DNA poly-

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merase-α, Ki-67 and the proliferating cell nuclear antigen (PCNA). SMC division and migration have been well characterized using [^{3}H]-thymidine in the rat carotid artery [5–10] and PCNA expression has been examined in excised rat aortae [24]. However, evaluation of BrdU uptake and PCNA expression in the perfusion-fixed rat carotid artery after balloon injury have not been previously reported. We examined the time course of BrdU incorporation and PCNA expression in the rat carotid artery injury model, compared the BrdU labeling to the degree of vessel wall injury and evaluated the antigenicity of α-actin in tissue processed for BrdU immunohistochemistry.

Materials and Methods

Surgery

All animal surgery was performed using guidelines established by the Seattle Veterans Affairs Medical Center and University of Washington Animal Care Committees. Forty male Sprague-Dawley rats weighing 400–425 g were given atropine (0.25 mg/kg) and anesthetized with intraperitoneal ketamine (75 mg/kg) and xylazine (5 mg/kg). Using sterile microsurgical technique, both common carotid arteries were exposed through a midline cervical incision. On both sides, a 2-Fr Fogarty catheter (American V. Mueller, Chicago, Ill., USA) was inserted via the external carotid artery, advanced to the proximal common carotid artery, inflated with 0.2 cm^3 of air and withdrawn 4 times [5]. Survival times after surgery were 1 (n = 6), 2 (n = 8), 3 (n = 5), 5 (n = 9), 10 (n = 5) and 20 (n = 4) days. An additional group of 3 animals served as uninjured controls. Rats were given 5-bromo-2'-deoxyuridine (Sigma, 150 mg/kg, dissolved in sterile saline) by intraperitoneal injection at 18, 12 and 6 h prior to perfusion. Fifteen minutes prior to perfusion, rats were anesthetized with pentobarbital (65 mg/kg) and given Evans blue (1.25 mg/kg) via intracardiac injection to verify the extent of arterial injury.

Perfusion-Fixation

Vessels were perfused at mean arterial pressure through a left ventricular cannula with 200 cm^3 warm heparinized (1 U/cm^3) lactated Ringer's saline followed by 200 cm^3 warm 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4). Both carotid arteries were immediately harvested and the Evans blue-stained segment of each vessel was equally divided for embedding in paraffin or plastic (below). A segment of small bowel was removed as a positive control. The tissue was immersion fixed in paraformaldehyde for 4 h, transferred to PBS and stored at 4°C. Vessel segments were digested en bloc in 1 *N* HCl for 30 min and embedded in either paraffin or glycol methacrylate (Energy Beam Sci., Agawam, Mass., USA). Paraffin cross-sections were cut at a thickness of 8 μm and plastic sections were cut at a thickness of 4 μm.

Immunohistochemistry

Paraffin sections were dewaxed, hydrated in graded ethanols and incubated with 0.3% hydrogen peroxide and 0.1% sodium azide to quench endogenous peroxidase activity. Sections were incubated with protease (Sigma Type XXVII, 7.8 U/ml) for 10 min and nonspecific background was blocked with 3% normal horse serum for 20 min. Slides were incubated with monoclonal anti-BrdU (Becton Dickinson, San José, Calif., USA) diluted 1:100 in PBS 1.0% BSA-3.0% normal serum for 2 h in a humidified chamber. Following incubation with biotinylated secondary antibody (Vector, Burlingame, Calif., USA, 1:200) and streptavidan-conjugated horseradish peroxidase (Jackson, Westgrove, Pa., USA, 1:5,000), slides were immersed in semidarkness in a solution of 0.025% 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 1.25 μl/ml of 3% hydrogen peroxide, then counterstained with nuclear fast red. Control slides of small bowel tissue and artery sections not exposed to primary antibody were run in parallel. Plastic sections were reacted at 37°C, with the anti-BrdU antibody diluted 1:50. The intensity of the stain was enhanced by poststaining incubation in 0.005% osmium tetroxide for 2 h.

PCNA staining was carried out on perfused tissue immersion fixed for 30 min and embedded in paraffin. Sections were air-dried overnight, blocked (as above), and reacted with clone PC10 (Dako, Carpinteria, Calif., USA) diluted 1:200 using the immunoperoxidase method. The tissue was counterstained with methyl green.

Actin staining was carried out on acid-denatured tissue by incubation with HHF35 specific for the α and γ isoforms (Dako) diluted 1:500 (plastic) or 1:1,000 (paraffin) in Tris-buffered saline containing 1.0% BSA and 3.0% normal goat serum. Sections were subsequently incubated with 5-nm-gold-conjugated goat antimouse antibody (Amersham, Arlington Heights, Ill., USA) diluted 1:50. Specimens were silver intensified by immersion in a solution of 0.025% silver nitrate and 0.05% hydroquinone in 0.2 *M* citrate buffer.

Analysis

For both paraffin and plastic sections, five or more vessel cross-sections separated by at least 40 μm were examined using a reticle and light microscopy at 40× magnification. Sections with apparent tissue loss during processing were excluded from analysis. The vessel wall was subdivided into two components; media (abluminal to the internal elastic lamina, IEL) and neointima (adluminal to the IEL). At early time points (control, 1–5 days), at least 1,000 total cells (primarily media) were counted and scored for each vessel. At the 10- and 20-day time points, a minimum of 1,000 cells as counted and scored for each compartment (media, neointima) of each vessel. For both immunoreactive BrdU and PCNA, nuclei containing the characteristic brown-black DAB deposits were scored as positive.

The labelling index (LI) for BrdU was expressed as the ratio of [chromogen-positive nuclei to total nuclei] × 100. For each time point, the LI for the media and neointima was expressed as mean ± SD. Comparisons of LI between plastic and paraffin-embedded tissue were performed using the Student's t test and a value of $p < 0.05$ was considered statistically significant. Relationship between cell number and BrdU labelling index for the media and neointima were determined using Spearman's rank correlation coefficient.

Results

Histology

Carotid artery cross sections after balloon injury showed a typical time course of SMC migration and proliferation as previously described [5–10, 25]. Within 48 h

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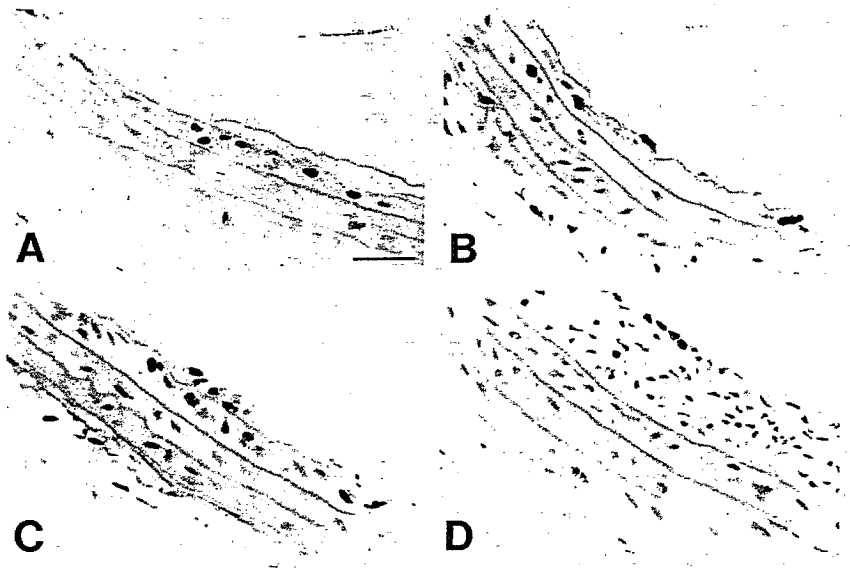

Fig. 1. Photomicrographs of rat carotid arteries stained for immunoreactive BrdU at various times after balloon catheter injury: (**A**) 48 h; (**B**) 72 h; (**C**) 5 days, and (**D**) 10 days. Bar = 10 μm.

of injury, there was evidence of complete endothelial desquamation with partial disruption of the internal elastic lamina (IEL) and variable degrees of medial damage (fig. 1). At 3 and 5 days after injury, occasional cells were noted adluminal to the IEL (fig. 1); neointimal cell number and area increased markedly at 10 and 20 days after injury (fig. 1).

BrdU and Actin Immunohistochemistry

Systemic distribution of BrdU was confirmed by intense staining of the intestinal epithelial crypt cells in all animals receiving the agent (fig. 2). The immunoperoxidase method produced high resolution of positive cells with minimal background staining and labeled nuclei were readily distinguishable even at low magnifications. Antigen in tissues embedded in paraffin and plastic appeared equally immunoreactive. Distribution of the nuclear chromogen was uniform in paraffin-embedded sections and granular in sections embedded in plastic (fig. 2). Determination of LI was easier in plastic sections compared to paraffin sections due to superior preservation of morphology in plastic at all time points.

Serial sectioning and staining for immunoreactive α-actin identified BrdU-labeled cells in the media and neointima as probable smooth muscle cells (fig. 3). Actin immunoreactivity persisted in tissue hydrolyzed with HCl.

Fig. 2. Photomicrographs of immunoreactive BrdU in small bowel intestinal crypt cells: (**A**) paraffin; (**B**) plastic. The chromogen is localized to crypt cell nuclei in a granular (plastic) or smooth (paraffin) pattern. Bar = 10 μm.

PCNA Immunohistochemistry

PC10 staining produced highly variable results with small changes in the postfixation time; optimal fixation times for perfused vascular and intestinal tissues did not correspond. To assess the effect of perfusion fixation by postfixation on PCNA labelling, we excised gut from a rat perfusion fixed with 4% paraformaldehyde. Pieces of gut

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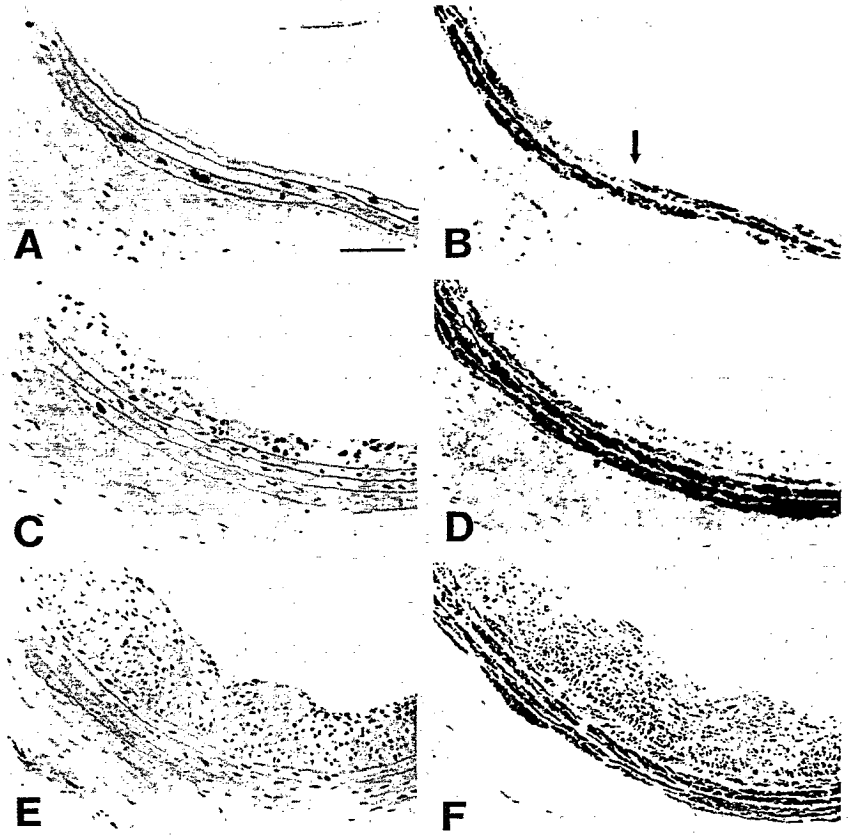

Fig. 3. Photomicrographs of serial carotid artery sections reacted for BrdU (**A, C, E**) or α-actin (**B, D, F**) at 48 h (**A, B**), 10 days (**C, D**) and 20 days (**E, F**) after balloon injury. Note the absence of α-actin immunoreactivity in the inner layers of the media at 48 h (arrow). Bar = 20 μm.

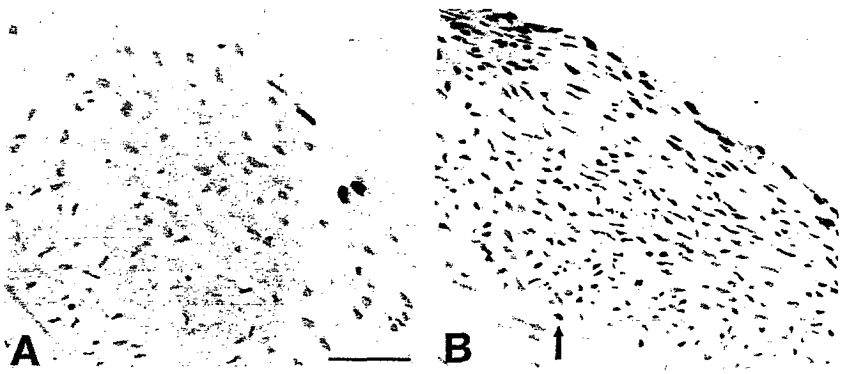

Fig. 4. Photomicrographs of serial carotid artery sections demonstrating immunoreactive BrdU (**A**) and PC10 (**B**) at 20 days following balloon injury. Cells in deep neointimal layers stain positive for PC 10 (arrow) but not BrdU. Bar = 10 μm.

were postfixed for time periods ranging from 30 min to 6 h. Optimal staining of crypt cells was seen at 30 min. With increased fixation time, specific staining decreased and nonspecific background staining (noncrypt cells and structures) increased. Of interest, leukocytes within the gut stained well at all fixation times, suggesting perhaps differential PCNA stability or populations in different cell types. Consequently, 'positive control' tissue could not be used to verify the sensitivity and specificity of immunostaining. Methyl green counterstaining did not appear to affect the LI determination for PCNA, as unstained sections demonstrated qualitative equivalent PCNA label-

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Table 1. BrdU labelling index in rat carotid artery after balloon catheter injury

Time	Paraffin			Plastic		
	n	media	neointima	n	media	neointima
Control	6	0.03±0.03	–	6	0.07±0.05	–
24 h	12	0.06±0.07	–	10	0.25±0.20*	–
48 h	11	19.93±11.63	–	14	9.42±6.30*	–
72 h	6	15.48±8.30	–	10	8.00±6.35	47.12±32.74
5 days	18	6.27±4.81	55.49±23.14	18	6.19±7.06	60.21±29.06
10 days	10	1.50±1.54	47.86±14.12	9	1.40±0.96	28.86±16.23
20 days	5	0.97±1.58	2.87±1.46	5	0.27±0.22	2.98±1.16

* $p < 0.05$.

ling to stained sections. There existed a wide range of staining intensity and chromogen distribution within labeled nuclei. The LI for PC10 generally exceeded the LI for BrdU as expected. If all labeled nuclei were scored positive, high growth fractions (~ 50%) were noted in neointimas as late as 20 days after injury (fig. 4).

Time Course of BrdU Labeling in SMC

Control vessels exhibited a negligible LI consistent with the low rate of DNA synthesis in uninjured arteries (table 1). Figure 5 shows the time course of BrdU labeling following balloon catheter injury in the media and neointima of paraffin- and plastic-embedded vessels. For both embedding matrices, the LI for BrdU in the media peaked at 48 h and was negligible beyond 5 days. Paraffin-embedded tissues demonstrated generally higher labeling indices compared to plastic-embedded tissues, and these differences were significant at three time points ($p < 0.05$). Neointimal cells adluminal to the IEL were first observed at 3 days and the mean LI for the neointima in both paraffin and plastic was greatest at 5 days. The number of neointimal cells per section increased progressively at 10 and 20 days after arterial injury. At late time points, BrdU labeling was generally limited to the luminal aspect of the neointima (fig. 4A).

Variability and Vessel Wall Injury

For a number of time points, there was considerable variability in the LI for BrdU. At 48 h, there was a direct relationship between the LI and the degree of medial injury as determined by the number of viable SMC per section (fig. 6A). In vessels with a high LI, regions of the innermost medial layer were devoid of cells and actin staining was greatly diminished (fig. 3). In contrast, vessels with a comparatively low LI had total SMC counts and actin-staining density comparable to control vessels.

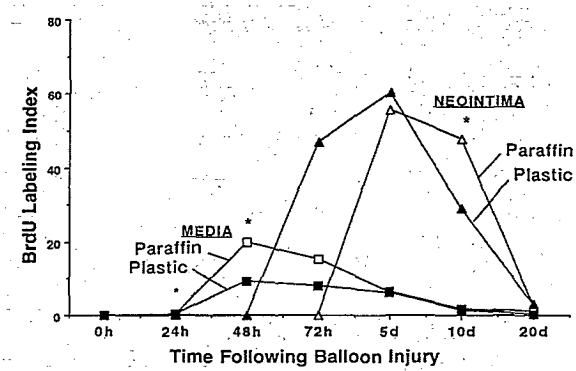

Fig. 5. Mean BrdU labeling index in the media and neointima at various times following balloon catheter injury to the rat carotid artery. * $p < 0.05$ paraffin vs. plastic.

At later time points, the percentage of cells staining for BrdU was considerably greater in neointimas with few cells compared to neointimas with high cell number. At 10 days the LI for the neointima correlated inversely with the mean total neointimal SMC number (fig. 6B). In essence, the number of BrdU-labeled cells along the luminal aspect of the artery was relatively consistent among vessels at 10 and 20 days: variations in the BrdU labellling index among animals were primarily due to variability in the number of cells present in the neointima.

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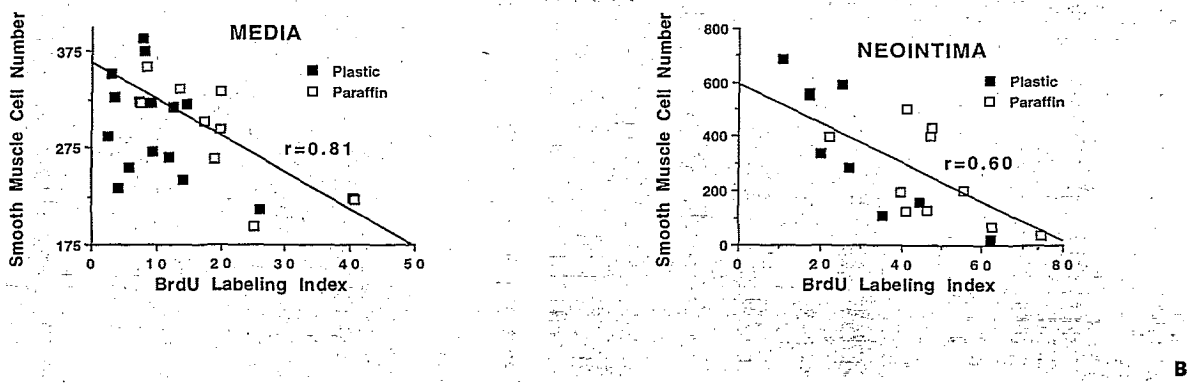

Fig. 6. Mean cross-sectional smooth muscle cell number and BrdU labeling index for individual rat carotid arteries embedded in paraffin (□): (**A**) media at 48 h after injury (r = 0.81); (**B**) neointima at 10 days after injury (r = 0.60).

Discussion

Endogenous Proliferation Markers

Antibodies have been developed against endogenous proliferation antigens such as PCNA, Ki67 and DNA polymerase-α. These markers are expressed in multiple phases of the cell cycle and presumably are present at the onset of proliferation prior to [^{3}H]-thymidine incorporation. The existence of these antigens obviates the need for administration of exogenous compounds and growth fractions obtained with these markers generally correlate well with other indices of cellular proliferation [26, 27].

In animal models of arterial injury, PCNA immunohistochemistry labels a greater proportion of proliferating SMC than either [^{3}H]-thymidine or BrdU [11, 24]. However, PCNA has a half life of approximately 20 h and a nonspecific nucleoplasmic form of the antigen persists in aldehyde-fixed tissue [28] leading to potential overestimates of the growth fraction. In addition, immunodetection of endogenous proteins is limited to those species exhibiting cross-reactivity and proliferation antigens tend to be labile and have fastidious fixation requirements. A number of factors have been shown to influence PCNA immunoreactivity, including fixation time [29–32], fixative composition [33, 34] and the source of antibody [33]. In addition, immunoreactive PCNA may be absent in certain proliferating neoplastic cells [35] and present in normal, nonproliferating tissues [36, 37]. In the current experiment, both fixation time and section thickness influenced PCNA immunoreactivity. Although PCNA has been employed to determine proliferative index in vascular tissue [24, 38], these data should be interpreted in light of potential variability for this technique.

Assessment of Growth Fraction by BrdU Immunohistochemistry

Although tritiated thymidine ([^{3}H]-thymidine) has proven an invaluable tool in the study of cytokinetics, use of radiolabeled precursors is accompanied by containment problems. Autoradiography and grain counting are laborious and time-consuming and the technique yields an unavoidable amount of background. Furthermore, [^{3}H]-thymidine may be radiotoxic to proliferating cells at certain doses [39].

Both [^{3}H]-thymidine and BrdU are incorporated into DNA by the salvage pathway [13]. The LI for BrdU correlates well with growth fractions derived from [^{3}H]-thymidine using a variety of cell and tissue preparations and protocols [14, 40–43]. Immunoreactive BrdU can be detected in tissues exposed to cross-linking fixatives [17] and antigenicity can be demonstrated in frozen specimens [44], plastic [44] and paraffin-embedded [17] tissues. The BrdU labeling method has been used previously to study vascular SMC proliferation in vivo [18, 19] and in vitro [45].

In this study, BrdU administered by serial injection over 18 h produced good systemic distribution as evidenced by strong immunoreactivity in proliferating intes-

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tinal crypt cells. Based upon estimates of SMC kinetics [12, 25], it seems likely that the majority of S-phase SMC were labeled during the exposure period. Although other investigators have coadministered BrdU and deoxycytidine in vivo [18, 19], we documented good BrdU incorporation and staining without deoxycytidine supplementation and noted no overt evidence of BrdU toxicity. Intestinal staining was appropriate with an absence of any clearly necrotic or polyploid cells and LIs in vascular tissue approximated values reported in [^{3}H]-thymidine studies.

BrdU Labeling Patterns in the Rat Carotid Artery Balloon Injury Model

The time course and magnitude of the BrdU incorporation in this model corresponded to that observed by others using [^{3}H]-thymidine [5–10, 25]. Other investigators have reported maximal [^{3}H]-thymidine growth fractions in the media of ballooned vessels at approximately 35 [25], 48 [6] and 96 h [8], and maximal medial values have ranged from approximately 25% at 35 h [25] to 43.5% at 4 days [8]. In the current experiment, there was a tendency for persistent high labeling rates at the luminal surface throughout the 20-day study period in agreement with autoradiographic cross-sectional [6, 10, 46] and Häutchen [6, 10] preparations. This pattern was most evident at 20 days when virtually all of the BrdU labeled cells were located at the luminal surface of the neointima. This observation suggests that persistent SMC proliferation in this model may be mediated by permeable serum factors.

BrdU Methodology

There was a significant variation in the LI for BrdU between plastic- and paraffin-embedded tissues, especially in the neointima. Virtually all intestinal crypt cells contained immunoreactive BrdU in our plastic embedded tissues corresponding to similar observations by others [47]. It therefore seems unlikely that BrdU antigenicity is altered by the embedding polymer. Antibodies may react only with antigens on the surface of plastic sections [48] and thinner sections might therefore exhibit higher LI. While we cannot exclude the possibility that methacrylate alters BrdU antigenicity, the favorable qualities of morphologic preservation and clarity of label make the plastic embedding method preferable except in conditions with low labeling indices (e.g. hypertension).

Actin

Most monoclonal antibodies to BrdU recognize BrdU only in single-stranded DNA, requiring harsh treatment of the tissue. However, other membranous and cytoplasmic epitopes retain antigenicity despite denaturation and proteolysis [47, 49]. In this experiment, acid hydrolysis (plastic) and acid hydrolysis plus protease digestion (paraffin) did not compromise α-actin immunoreactivity and localization of the antigen was comparable to the pattern seen in nondenatured control tissue. BrdU-labeled SMC in the media stained for α-actin at early time points, whereas BrdU-labeled neointimal cells generally stained poorly or not at all. Actin antigenicity in the neointima was diminished at 10 days after injury for both BrdU-labeled and unlabeled cells and was independent of processing technique. In contrast, medial cells remained highly reactive for α-actin at this time point. Neointimal α-actin immunoreactivity returned at 20 days with a well-defined pattern in deep neointimal layers and a diffuse pattern at the luminal surface. This reversible phenotype alteration and time course are in agreement with the findings of injury-induced smooth muscle de-differentiation reported by others [50] in which SMC express alterante isoforms of actin during synthetic (β and γ isoforms) and contractile (α isoform) phases.

Variability and Extent of Arterial Injury

Determinations of mean LI for BrdU observed in this experiment were consistent with prior observations [5–10, 25]. However, we noted considerable variability among individual vessels most likely related to differences in the extent of injury to the vessel wall. Cross-sectional and longitudinal variability in SMC hyperplasia has been observed previously [46]. Although others [51] have noted no relationship between the severity of injury and SMC hyperplasia, several investigators have attributed inconsistency of hyperplasia to small variations in the standard balloon technique [52, 53]. In our study, variability of the LI for BrdU at 48 h appeared to be a function of the magnitude of injury manifest by differences in SMC necrosis and loss of α-actin.

Variability of the migratory response was also evident in this study and has been noted previously [8]. While virtually all vessels had a hypercellular innermost media at 5 days, not all vessels exhibited neointimal cells at this time. Neointimal formation is dependent upon migration of both dividing and nondividing SMC [7] and it is therefore conceivable that injury-induced alterations of migration also contribute to variable neointimal cell numbers and LI. There is a threshold of intimal damage required for the initiation of SMC hyperplasia [54] and the magnitude and duration of endothelial injury have a major impact upon subsequent SMC proliferation [52]. Our findings confirm

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other reports supporting a key role for medial damage in neointimal formation [55, 56] and clinical complications secondary to arterial injury [57]. An alternate explanation for the variability of the vascular response may be the presence of SMC heterogeneity [7, 9, 12, 50, 58] and the inadvertant preservation or deletion of specific populations as a result of balloon injury. BrdU labeling appears to correlate best with indices of medial vascular damage evident at 48 h after injury.

Conclusions

The time course and magnitude of BrdU uptake in the balloon catheter-injured rat carotid approximate the patterns reported in studies utilizing [^{3}H]-thymidine. BrdU can be detected in both paraffin and plastic embedded tissues, although histologic preservation was superior in plastic embedded specimens. Variability of medial and neointimal LI for BrdU between vessels at single time points following injury appears to be related to the severity of the inury. BrdU incorporation was inversely related to actin expression in the neointima. In our application, immunodetection of BrdU appears to be preferable to PCNA for the assessment of SMC proliferation.

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